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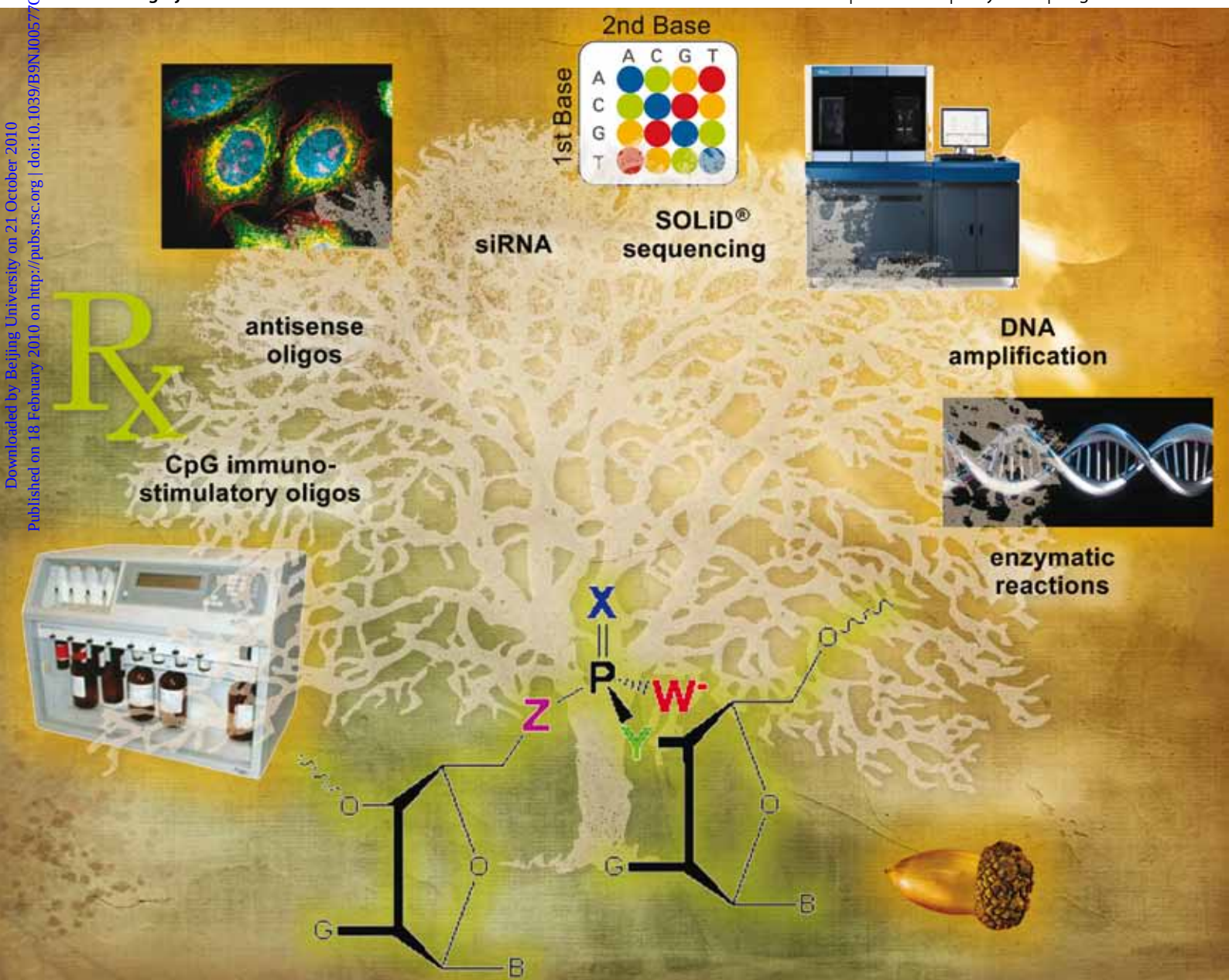


## Themed Issue: Biophosphates

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**PERSPECTIVE**
Gerald Zon
Automated synthesis of phosphorus–sulfur analogs of nucleic acids—25 years on: potential therapeutic agents and proven utility in biotechnology

# Automated synthesis of phosphorus–sulfur analogs of nucleic acids—25 years on: potential therapeutic agents and proven utility in biotechnology†

**Gerald Zon***

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Replacement of non-bridging oxygen by sulfur in one or more internucleotide linkages in synthetic oligodeoxynucleotides (ODNs) was first fully automated in 1984. The present account highlights how ready access to these nuclease-resistant, phosphorothioate-modified oligodeoxynucleotides (PS-ODNs) and chemically related compounds, notably phosphorothiolates, has enabled remarkably broad advances across basic science, medical research, and biotechnology. These developments exemplify how an initially narrow-scope chemical reaction—in this case, automated sulfurization of an internucleoside phosphite—can give rise to diverse applications far beyond initial expectations.

## 1. Introduction

Ralph Waldo Emerson, an American essayist, philosopher and poet, noted that "*the creation of a thousand forests is in one acorn*" ("History" Essays, First Series, 1841). From what follows, it will hopefully be apparent that this truism can be reflected in the case wherein many practical uses derive from a single chemical reaction. The present account traces growth of applications arising from fully automated solid-phase synthesis of PS-ODNs by sulfurization of an internucleoside phosphite with elemental sulfur first reported in 1984 by Stec *et al.*[1] Manual versions of this approach were published around the same time by Eckstein and coworkers,[2] who had previously found that $S_P$ and $R_P$ (Fig. 1) PS-linkages exhibited stereo-dependent resistance to cleavage by nucleases[2] (phosphite sulfurization was reported[3] much earlier for an RNA dimer). Following the automated synthesis of novel PS-ODNs having a completely modified thiophosphate-backbone,[1] it was postulated

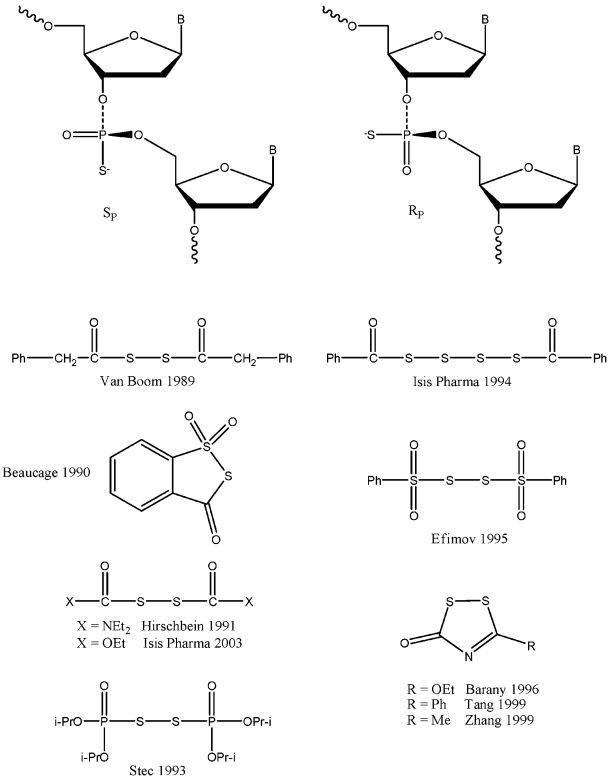


**Fig. 1** Chemical structures of internucleotide $S_P$ and $R_P$ diastereomeric phosphorothioate linkages and reported sulfurizing reagents for automated synthesis of these moieties (Van Boom;[12] Beaucage;[13] Hirschbein;[14] Isis Pharma (2003);[15] Stec;[16] Isis Pharma (1994);[17] Efimov;[18] Barany;[19] Tang;[20] Zhang[21]).

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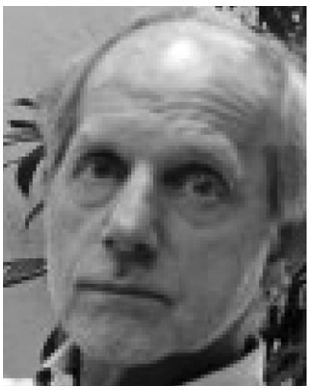
† Dedicated to Professor Wojciech J. Stec on the occasion of his 70th birthday. This article is part of a themed issue on Biophosphates.



**Gerald Zon**

*Gerald Zon (PhD Princeton Univ. 1971) developed automated synthesis of DNA analogs at NIH, and then at Applied Biosystems (ABI). He co-founded Lynx Therapeutics as an antisense company in 1992, and returned to ABI in 1999 to head DNA sequencing R&D. Currently a Research Fellow at Life Technologies, he works on novel DNA sequencing approaches. In 2006 he was admitted as a Fellow of The Royal Society of Chemistry.*

and found by several collaborating laboratories at the National Institutes of Health (NIH) that these compounds represented a new class of long-lived antisense agents for inhibition of gene expression.[4–7] Unexpected observations in these and related exploratory studies of fully modified PS-ONDs included sequence-independent antiviral,[6] immunostimulatory[8] and cytotoxic[9] effects. Nevertheless, increased commercial interest in developing antisense-based therapeutics during these early years led to numerous investigations of alternative ODN backbone chemistries, chimeric structures having mixed backbone and/or sugar modifications, scale-up of synthesis, pharmacokinetics and toxicology.[10,11]

Initial commercialization of automated PS-ODN synthesis by Applied Biosystems in 1988 involved one-step sulfurization of a support-bound oligo(hydrogen-phosphonate) backbone with elemental sulfur, which was particularly useful for $^{35}$S-radiolabeling.[22] However, this hydrogen-phosphonate methodology was not flexible, compared to phosphoramidite methodology, for providing mixed-backbone constructs such as DNA/2′-*O*-methyl-RNA chimera that became of interest as possibly new and improved antisense constructs. Considerable effort was therefore directed at increasing efficiency of internucleoside-phosphite sulfurization—and decreasing cost—by replacement of elemental sulfur with alternative sulfur-donor reagents such as those shown in Fig. 1.[12,13,15–21] Beaucage[13] reagent continues to be popular for small, research-scale synthesis whereas large, commercial-scale production uses other sulfur-donors. Somewhat ironically, a recent report[23] by Isis Pharmaceuticals concluded that use of phenylacetyl disulfide (PADS) first reported in 1989 by Van Boom and coworkers[12] provides >99.9% step-wise efficiency of sulfurization and is superior for commercial-scale manufacturing.

Among new backbone chemistry for modified ODNs that are accessible by automated solid-phase synthesis, achiral 3′- and 5′-phosphorothiolate (Fig. 2) congeners of chiral PS-ODNs were originally investigated by the Cosstick[24] and Engels[25] laboratories, respectively, using corresponding 3′- and 5′-thiophosphoramidites. While 3′- and 5′-phosphorothiolate-modified ODNs were not widely adopted as antisense agents, reported[24,25] facile and specific cleavage of the P–S bond by silver ion would be later found by others to have extraordinarily great value, scientifically and commercially, in a completely different context—DNA sequencing—as discussed later in this account.

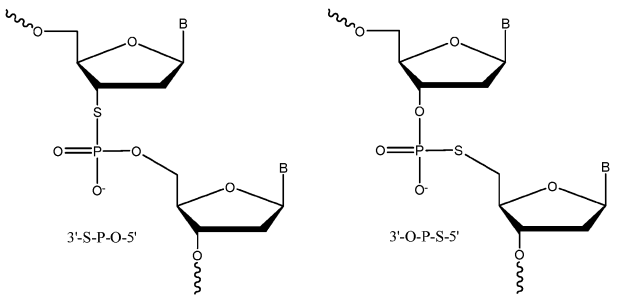



**Fig. 2** Chemical structures of internucleotide 3′-S–P–O-5′ and 3′-O–P–S-5′ phosphorothiolate linkages.

## 2. PS-modified oligonucleotides

### 2.1 Numerical survey of antisense and related applications in journal articles and patent publications

Chemical Abstracts Service (CAS), a division of the American Chemical Society, offers a pay-per-search web-based tool (SciFinder) for analyzing references from more than 10 000 journals and 59 patent authorities. In September 2009, CAS SciFinder was used to search for "phosphorothioate" in all references published during 1985–2008. The resultant references in journals and patents were each further searched for "antisense," and the items found were then sorted by year to give the results shown in Fig. 3. These numerical data indicate gradual growth of journal publications dealing with antisense phosphorothioates until the late 1990s followed by a fall-off. The relatively large numbers of such patents, which peak somewhat later in time, attest to potential commercial utility. The trend in journal publications found by CAS SciFinder was independently checked by carrying out a similar web-based search using PubMed, which is freely provided by the joint U.S. National Library of Medicine-NIH National Center for Biotechnology Information. PubMed focuses on articles in life-science, with a concentration on biomedicine, derived from 5200 journals. PubMed content thus contrasts with that accessed by SciFinder in two ways: PubMed is not concentrated in chemistry and it excludes patents. The PubMed results shown in Fig. 3 closely parallel the non-patent content from CAS SciFinder but with fewer articles in PubMed that is consistent with there being fewer references in chemistry-oriented journals in PubMed.

In the introduction it was noted that early investigations of fully modified PS-ODNs as possible antisense agents led to observation of immunogenicity[8] and indications of avid binding to certain proteins.[26,27] These and related findings led to numerous follow-on investigations and developments, as is evident from the combined journal and patent publications shown in Fig. 4 that were found in CAS SciFinder by sub-searching "phosphorothioate" using the prefix "immuno" or "aptamer." Fig. 4 also reveals a more recent burst of publications beginning in 2003 found by similarly sub-searching "phosphorothioate" using the term "siRNA," *i.e.*, PS-modified short-interfering RNA (siRNA) employed for inhibition of gene expression. These three categories of applications and

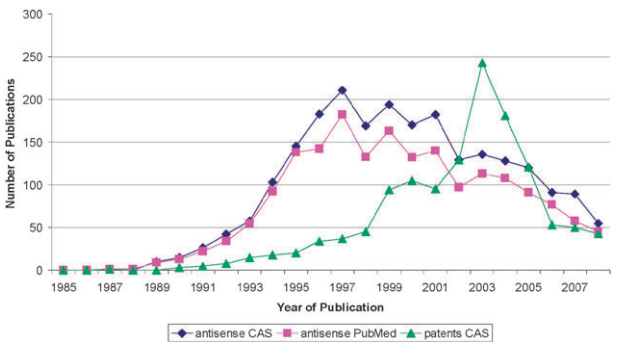



**Fig. 3** Graph showing annual number of publications indexed to antisense phosphorothioate oligonucleotides in journals (CAS or PubMed) or patents (CAS).

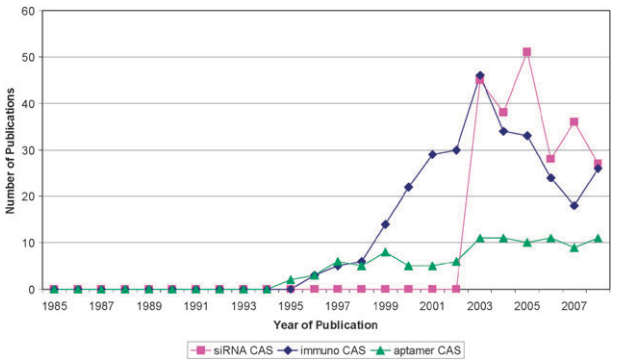



**Fig. 4** Graph showing annual number of publications indexed to immunostimulatory or aptameric or siRNA phosphorothioate oligonucleotides in journals and patents (CAS).

several other new uses, which will be briefly discussed in what follows, collectively suggest continued utility of PS-modified oligonucleotides in years to come.

### 2.2 Current status of clinical development of PS-modified oligonucleotides

Early enthusiasm for first-generation antisense oligonucleotides completely modified with PS linkages waned as problematic sequence-independent "backbone" effects were found, along with less than favorable pharmacokinetics, cellular uptake, and potency.[28] Nevertheless, many investigations aimed at mitigating these problems eventually led to improved classes of antisense compounds wherein fewer PS linkages are used in conjunction with addition of other modified sugar and/or base moieties (for an early review, see Cook[29]). Resultant second- or third-generation compounds include, for example, 2′-*O*-methoxyethyl (MOE) or LNA modifications. Table 1 provides a list of selected antisense compounds modified with PS linkages that are in clinical development sponsored by various companies as of September 2009. Although discussion of the information in Table 1 is beyond the scope of the present account, it should be evident that there is very substantial effort being devoted to these clinical investigations.

In addition to obtaining further information from journal publications, patents, and company web sites, the interested reader is encouraged to take advantage of extensive on-line information available at http://clinicaltrials.gov. This web site is a registry of federally and privately supported clinical trials conducted in the United States and around the world. ClinicalTrials.gov gives information about a trial's purpose, who may participate, locations, and phone numbers for obtaining more details. A mapping tool at this web site allows one to search and visualize the global distribution and numbers of clinical trials on a country basis. A search for "antisense" in ClinicalTrials.gov in September 2009 provided the global distribution map in Fig. 5, which also depicts how one can click on a country or sub-region of interest to obtain further detail. Additional information that can thus be obtained includes clinical status (*e.g.* "recruiting" or "active, not recruiting" or "completed" or "withdrawn" *etc.*) and a link to pertinent details (*e.g.*, sponsor, drug(s), intervention target, disease or condition, phase, primary and secondary outcomes, detailed description, *etc.*). What is striking about Fig. 5 is the relatively large number of antisense clinical trials involving many countries. An optimist would assume that, given the extent and diversity of these efforts that include the compounds in Table 1, PS-modified antisense drugs will be found to have clinical utility.

### 2.3 Brief survey of PS-modified immunostimulatory oligonucleotides and siRNA

Early preclinical toxicology studies[8] of mice given i.v. injections of CpG-containing PS-ODNs led to observations analogous to murine B-cell immunostimulatory effects subsequently investigated in detail by Krieg *et al.*[30] using synthetic oligonucleotides having CpG motifs without or with PS linkages. In the later work, it was found that CpG PS-ODNs stimulated B cells at concentrations more than 2 logs lower than those required with unmodified, phosphodiester

**Table 1** Selected companies, in alphabetical order, and PS-modified antisense compounds in clinical development

| Company | Compound | Target | Disease or indication | Clinical phase |
|---|---|---|---|---|
| Aegera | AEG-35156 | X-linked inhibitor of apoptosis XIAP | Cancer | 1 |
| Antisense Pharma | AP 12009 | TGF-β 2 | Cancer | 1/2 |
| Genta | Genasense | Bcl-2 | Solid tumors, blood cancers | 3 |
| | G4460 | c-Myb | Solid tumors | 1 |
| Geron | GRN163L | Telomerase (template antagonist) | Solid tumors, blood cancers, NSCL cancer | 1 |
| Isis Pharmaceuticals | ISIS 301012 | ApoB-100 | High cholesterol | 3 |
| | ISIS 113715 | Protein tyrosine phosphatase 1B | Diabetes | 2 |
| | ISIS-CRPRx | C-reactive protein | CAD, inflammation, renal | 1 |
| | LY2181308 | Survivin | Cancer | 2 |
| | LY2275796 | eIF-4E | Cancer | 1 |
| Lorus Therapeutics | GTI-2040 | R2 subunit of ribonucleotide reductase | Renal cell carcinoma, AML | 2 |
| | GTI-2501 | R1 subunit of ribonucleotide reductase | Prostate and kidney tumors | 1/2 |
| MethylGene | MG98 | Human DNA methyltransferase 1 | Solid tumors | 1 |
| Neopharm | LErafAON | c-Raf | Solid tumors | 1 |
| Oncogenex | OGX-011 | Clusterin | Prostate and breast cancer, NSCL cancer | |
| | OGX-427 | Heat-shock protein Hsp27 | Cancer | 1 |
| Prosensa | PRO051 | Exon-51 skipping in dystrophin gene | Duchenne muscular dystrophy | 1/2 |
| Santaris Pharma | SPC2996 | Bcl-2 | Cancer | 1/2 |
| | SPC3042 | Survivin | Cancer | 1 |
| Topigen | TPI-ASM8 | Chemokine receptor-3/IL3,5/GM-CSF | Asthma | 2 |
| | TPI-1100 | Phosphodiesterases PDE4 and PDE7 | COPD | Pre-IND |

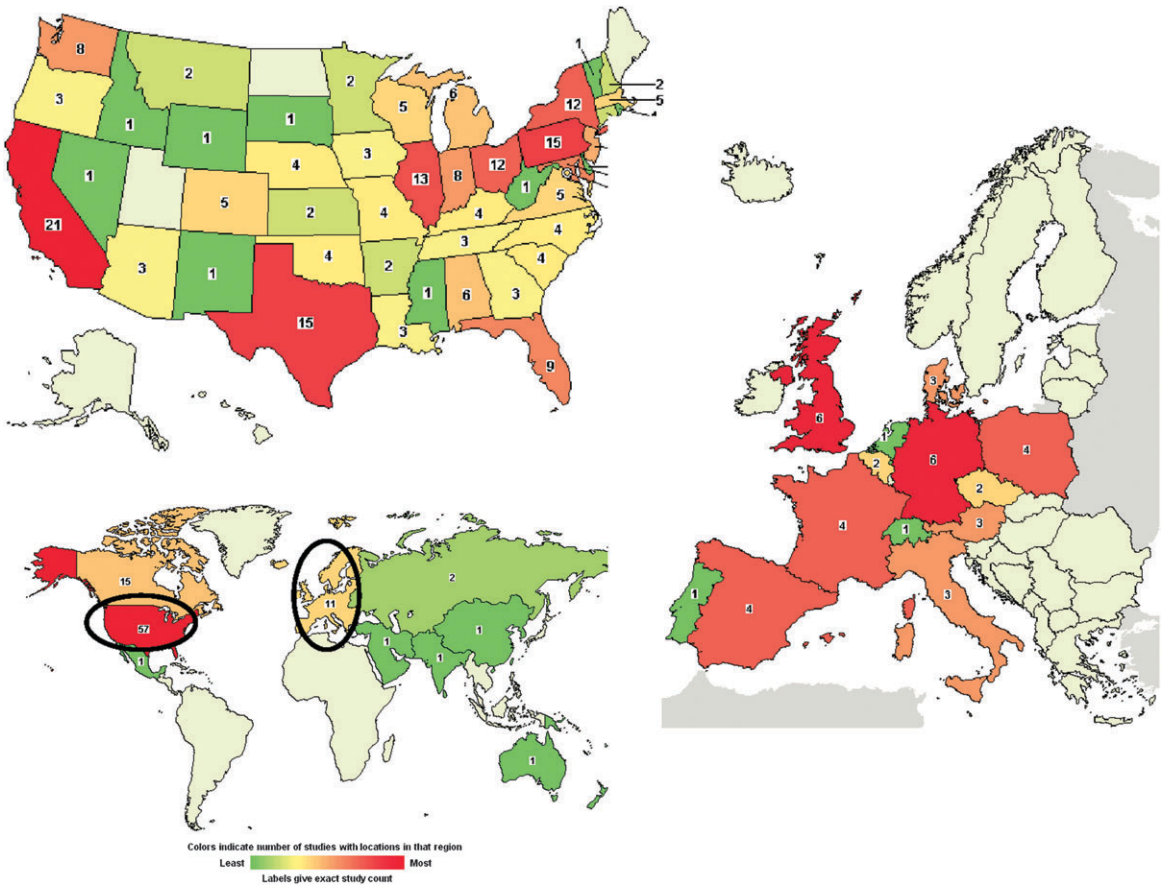



**Fig. 5** Maps taken from ClinicalTrials.gov in September 2009 showing location and number (with color scale) of antisense clinical investigations. Circles exemplify selection of regions (USA and Europe) that can each be expanded to obtain more detailed maps (top and right, respectively), which in turn can be used to obtain further detail (not shown).

oligonucleotides. It was suggested[30] that "immune-stimulating CpG oligonucleotides may prove clinically useful as adjuvants and biological response modifiers." Since that time, and as shown in Fig. 4, there have been numerous scientific and patent publications dealing with immunostimulatory properties of PS-modified oligonucleotides. The following commercial efforts are aimed at demonstrating clinical utility of such compounds.

• Coley Pharmaceutical Group, which has extensive publications and patents on CpG oligonucleotides and was acquired by Pfizer in 2007, developed a fully phosphorothioated 32-mer called CPG 7909.[31] In September 2009 there were 37 studies found for CPG 7909 at ClinicalTrials.gov (see section 2.2).

• Dynavax Technologies has developed immunostimulatory CpG DNA sequences (ISS) that include a PS-stabilized 22-mer oligonucleotide called 1018 ISS for treatment of genital herpes simplex virus type 2 (HSV-2).[32] Information in ClinicalTrials.gov in September 2009 was available for 11 studies by Dynavax.

• Idera Pharmaceuticals has recently published results of structure–activity studies of CpG oligonucleotides having fully phosphorothioated backbones.[33] There were 4 studies in ClinicalTrials.gov found for Idera in September 2009.

RNA interference (RNAi) by siRNA is now widely recognized as involving incorporation of the antisense strand of these antisense/sense double-stranded RNA structures into the multi-protein RNA-induced silencing complex (RISC), which then cleaves target mRNA. Among the initial flurry of publications in 2003 (see Fig. 4) dealing with siRNA and phosphorothioates, there were several reports[34–36] that investigated the effects of location and number of PS linkages in siRNA. Chiu and Rana[35] found that PS modifications could increase siRNA stability, albeit with some loss of activity, and suggested that "stabilized phosphate linkages was a viable option for prolonging RNAi effects." Tuschl and coworkers[36] reported that RNase-protecting PS and 2′-fluoropyrimidine RNA backbone modifications of siRNA did not significantly affect silencing efficiency, although cytotoxic effects were observed when every second phosphate of a siRNA duplex was replaced by PS. Interestingly, Corey and coworkers[34] found that fully phosphothioated ssRNA 21-mers were degraded in serum whereas double-stranded siRNA duplexes with PS modifications were stable for incubation in serum up to 72 h. In addition, RNAi activity was largely retained using siRNA with limited terminal modification by PS linkages. Following these early studies, investigators at Isis Pharmaceuticals reported[37] a comprehensive, systematic evaluation of PS content and positional effects of 2′-sugar modifications (2′-fluoro, 2′-*O*-methyl, and 2′-*O*-MOE) in the antisense and sense strands of siRNA in HeLa cells.

More recently, Alnylam Pharmaceuticals and collaborators reported[38] siRNA-mediated RNAi in rodents and nonhuman primates targeting an enzyme (PCSK9) that regulates low density lipoprotein receptor protein levels and function to validate PCSK9-lowering agents as a future strategy for treatment of hypercholesterolemia. A series of ~150 cross-species siRNAs were designed—including avoidance of immune stimulatory sequence motifs—and screened *in vitro* to obtain four candidate compounds for further evaluation. These candidate siRNAs each had a single PS linkage at the 3′ end of both the sense and antisense strand, in addition to 2′-*O*-methyl modifications at certain positions.

A search of ClinicalTrials.gov (see section 2.2) for siRNA in September 2009 led to information concerning 14 clinical trials, although it was not certain from the information available whether any of these studies included siRNAs modified with PS. In any case, commercial sponsors that were listed included, in alphabetical order, Allergan (2 studies), Calando Pharmaceuticals, Opko Health Inc. (3 studies), Quark Pharmaceuticals, Silence Therapeutics AG, Sirna Therapeutics Inc., and Tekmira Pharmaceuticals Corp.; the remaining sponsors were non-commercial.

### 2.4 Intracellular mRNA monitoring in living cells

The possibility of using fluorescence resonance energy transfer (FRET) to detect and quantify nucleic acid hybridization in living cells was apparently first described in 1988 by Zamecnik and coworkers.[39] However, their two-probe approach involving adjacent hybridization of appropriately dye-labeled oligonucleotides to mRNA was not widely investigated, relative to use of "molecular beacons" (MBs) that were later introduced by Tyagi and Kramer[40] (1996) for improved FRET-based detection of nucleic acids in general. The following quote taken from that paper[40] was indeed prescient as there have been a growing number of publications dealing with chemically modified, nuclease-resistant MBs in living cells.

"*Molecular beacons should also be suitable for the detection of specific nucleic acids within living cells. Although the presence of the fluorophore and quencher should protect molecular beacons against degradation by exonucleases, it may be necessary to modify the nucleotides or the internucleotide bonds to provide protection from endonuclease. Since there is no need to remove unhybridized probes prior to viewing fluorescence, molecular beacons can serve as vital stains, enabling the origin, movement, and fate of specific mRNAs to be traced.*"

In addition to MBs having nuclease-resistant 2′-*O*-methyl and LNA modifications, incorporation of PS linkages has been reported[41] for monitoring p21 mRNA in tumor cells, following doxorubicin-induction of p21, as a model system to assess whether MBs could be used to monitor p21 production that portends poor patient prognosis and poor response to chemotherapy. Based on the results obtained, it was concluded that p21 activation following exposure to chemotherapy could be imaged in cells using PS-modified MB methodology, although there was a relatively narrow window for dose *versus* detectable signal. More recently, the MB depicted in Fig. 6 having 2′-*O*-methyl and PS linkages was successfully used for

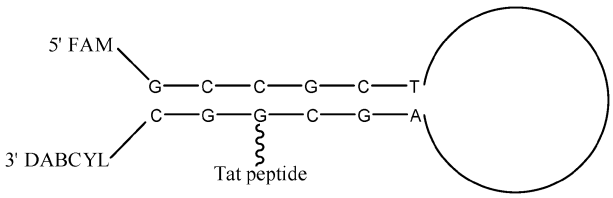



**Fig. 6** MB having a double-stranded stem and single-stranded loop structure comprised of 2′-*O*-methyl RNA and PS linkages throughout, except for a thiol-dG moiety, which is conjugated to Tat peptide through a maleimide bridge.[42] End-labeling employs 3′ fluorescein (FAM) and 5′ 4-(4-dimethylaminophenylazo)benzoic acid (DABCYL).

real-time detection of coxsackievirus B6 replication in living Buffalo green monkey kidney cells *via* Tat peptide delivery.[42]

Inadequate sensitivity of MBs, regardless of structural details, has been reported by others to be a limitation for monitoring low copy-number mRNA. This has prompted innovative approaches using enzymatic signal amplification of MBs following hybridization to mRNA targets,[43] as well as non-MB strategies[44] for probe-design to obtain higher signal-levels through, for example, mRNA-mediated "turnover" of probes to significantly increase (*e.g.*, ~100-fold[44]) fluorescence.

Non-optical methods providing increased sensitivity include use of radiolabels. In conjunction with development of new methods for positron emission tomography (PET) scans, biodistribution of a $^{68}$Ga-labeled 17-mer PS-ODN complementary to human mutant K-ras oncogene has been imaged in rats[45] (see also Roivainen *et al.*[46]). Unfortunately, no data were reported for specificity toward mutant *vs.* normal K-ras mRNA. On the other hand, specificity of detection was demonstrated in another study[47] using a $^{99m}$Tc-radiolabeled 18-mer PS-ODN targeting human telomerase reverse transcriptase (hTERT), which is present in most malignant cells but is undetectable in most normal somatic cells. Following i.v. injection of antisense or sense PS-ODN constructs in mammary tumor-bearing mice, biodistribution and *in vivo* imaging was performed periodically. In comparison with the sense compound, the antisense PS-ODN specifically inhibited expression. Moreover, tumor radioactivity uptake of the antisense PS-ODN was significantly higher than that of the sense oligonucleotide ($P < 0.05$). Finally, hTERT-expressing xenografts were clearly imaged at 4–8 h non-invasively after injection of the antisense PS-ODN, whereas xenografts were not imaged at any time after injection of the sense ODN.

*In vivo* imaging of gene transcripts in live animals without using radiolabels has been recently demonstrated by use of PS-ODNs and NMR.[48] To circumvent limitations of using postmortem brain in molecular assays, avidin–biotin binding was used to couple superparamagnetic iron oxide nanoparticles (SPIONs) (15–20 nm) to PS-ODNs complementary to c-fos and β-actin mRNA (SPION-cfos and SPION-β-actin, respectively) for *in vivo* magnetic resonance imaging (MRI) to monitor gene transcription. These complexes were retained at least 1 d after intracerebroventricular infusion into mice. SPION retention measured by MRI was compared with histology of iron oxide (Prussian blue) and fluorescently labeled PS-ODN. Retention of SPION-cfos and SPION-β-actin positively correlated with c-fos and β-actin mRNA maps obtained from *in situ*

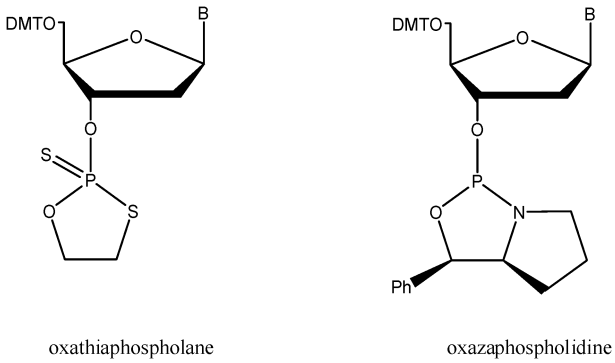
hybridization. Furthermore, in animals that were administered SPION-cfos and amphetamine, retention was significantly elevated in expected regions of the brain. Control groups that received SPION-cfos and saline or that received a SPION conjugate with a random-sequence PS-ODN and amphetamine showed no retention.

### 2.5 DNA polymerase-mediated PCR, whole genome amplification (WGA) and single-base extension (SBE)

Use of a single PS linkage at the 3′ end of primers to block 3′ to 5′ exonuclease activity of thermostable polymerases for improved PCR results was first described by Skerra[49] in 1992. More recently, this chemically simple yet remarkably useful PCR primer design-strategy has been extended to whole genome amplification (WGA). In contrast to PCR amplification, which uses specific sequences of forward and reverse primers that are complementary to specific regions of DNA, WGA employs so-called "random" (*i.e.*, degenerately synthesized) oligonucleotide primers. A 15-mer random ODN primer was successfully used for PCR amplification of a large fraction of DNA sequences present in a single human sperm cell, while use of a 6-mer random ODN primer was not successful under a variety of conditions.[50] In later work by others[51,52] concerning isothermal rolling-circle amplification (RCA) with Phi29 polymerase using random 6-mers, ODN primer degradation by Phi29 exonuclease activity was demonstrated, and then mitigated by utilizing two 3′ terminal PS linkages. This publication[51] and companion work[53] demonstrating less than 3-fold bias of amplification—in contrast to 4–6 orders of magnitude for PCR-based WGA—have been collectively cited in ~600 publications since 2001–2002, based on a search in CAS SciFinder in September 2009. This attests to widespread utility of Phi29 amplification of DNA that is enabled by blocking exonuclease activity by simply incorporating a couple of polymerase-compatible 3′ terminal PS modifications in random primers.

Single-base extension (SBE) of primers using DNA polymerases and fluorescently or mass labeled 2′,3′-dideoxynucleotide 5′-triphosphates (or other terminators) underlies various different assay-formats for detection of base mutations or single-nucleotide polymorphisms (SNPs). Array-based SNP genotyping in turn enables clinically relevant genome-wide association studies for disease and risk factors that are of widespread interest. Di Giusto and King[54] were first to demonstrate that SBE using proofreading (exo$^+$) DNA polymerases and primers with one PS linkage at the 3′ end reduced the level of undesired misincorporation. This use of proofreading polymerases and primers with a 3′ terminal PS linkage as a simple and cost-effective means to improve fidelity in a range of single-substrate SBE assay formats has been patented.[55] The influence of chirality of diastereomeric PS linkages in primers for SBE with proofreading DNA polymerases was recently investigated by Nawrot, Paul and coworkers,[56] who used Stec's oxathiaphospholane (Fig. 7) (OTP) method[57] for synthesis of a primer having a diastereomerically pure $R_P$ or $S_P$ 3′ terminal PS linkage.

Regarding stereoselective synthesis of chiral PS-ODNs, which was first achieved using Stec's OTP method employing strong organic base-catalyzed coupling,[57] oxazaphospholidine (Fig. 7) derivatives have been recently reported as more conventional, phosphoramidite-like monomer units for acid-catalyzed coupling.[58] These oxazaphospholidines are configurationally stable at phosphorus, and their diastereomeric purity was not compromised by epimerization in the presence of an acidic activator during coupling on a solid support. Inhibition of epimerization at phosphorus was attributed to ring-strain effects, and formation of both $R_P$ and $S_P$ PS-linkages occurred with high diastereoselectivity (≥99%).




oxathiaphospholane oxazaphospholidine

**Fig. 7** Chemical structures of nucleoside 5′-*O*-dimethoxytrityl (DMT) 3′-*O*-(2-thio-1,3,2-oxathiaphospholane) and 3′-*O*-(1,3,2-oxazaphospholidine) derivatives.

Incorporation of $R_P$ or $S_P$ phosphorothioates at the cleavage site in ribozymes has been widely employed to elucidate the mechanistic role of $Mg^{2+}$ ions. Compared to the $S_P$ diastereomer that cleaves well, the $R_P$ isomer reacts much slower but its rate of cleavage can be increased by use of $Mn^{2+}$ ions. This so-called "rescue" effect has been ascribed to the relative preference of divalent metal ions to bind to oxygen and sulfur.[59] However, other effects have been discussed.[59,60]

### 2.6 Brief survey of "thioaptamers"

Avid binding of PS-ODNs to proteins, which has been known for some time,[26,27,61] has led to interest in use of PS-ODNs as decoys[62] or aptamers.[63] In addition to automated synthesis of PS-modified ODNs as single sequences or "pools" *via* mixed-couplings, PCR-based synthetic approaches offer certain advantages. Gorenstein and coworkers[63] described the later approach for construction and screening of PS-DNA aptamers that bind tightly to the nuclear factor for human IL6 (NF-IL6), a basic leucine zipper transcription factor involved in the induction of acute-phase responsive and cytokine gene promoters in response to inflammation. Using a random combinatorial selection approach and PCR-compatible α-thio dNTPs for amplification, they obtained specific 22-mer thiophosphate backbone substitution sequences (at dA positions only) with nanomolar binding to NF-IL6. A variation of this strategy involves use of systematic evolution of ligands by exponential enrichment (SELEX) to derive a phosphodiester aptamer that is then improved by incorporation of phosphorothioate linkages. For example, a patent application[64] by Archemix Corp. describes modifying single-stranded aptameric oligonucleotides to improve binding to IL-23, to IgE, and to von Willebrand factor that were each identified and

optimized by SELEX. The affinity of these aptamers for their respective targets was further increased by replacing one phosphodiester linkage with a PS linkage.

A new and particularly exciting application of aptamers involves targeting prions. Although transmissible spongiform encephalopathies (TSEs) are incurable, a key therapeutic approach is prevention of conversion of the normal, protease-sensitive form of prion protein (PrP-sen) to the disease-specific protease-resistant form of prion protein (PrP-res). Investigators at the NIH reported[65] use of degenerate PS-ODNs as low-nM PrP-res conversion inhibitors with strong antiscrapie activities *in vivo*. They noted that hydrophobicity and size were important, while base composition was only minimally influential. Moreover, prophylactic treatments with these compounds more than tripled scrapie survival periods in mice. It was suggested that these antiscrapie activities, and much lower anticoagulant activities than that of pentosan polysulfate, are favorable indications for possible use of PS-ODNs as new compounds for the treatment of TSEs.

"Thioaptamer Proteomics Chips" recently described by Gorenstein and coworkers[66,67] use an automated method of creating split-synthesis bead-libraries, along with bead-based high-throughput screening of thioaptamer bead-libraries, to rapidly select thioaptamers for the development of a thioaptamer-based proteomics array. Such arrays, in conjunction with mass spectrometry, were said to enable identification and quantification of proteins and protein complexes associated with critical signaling and immune response pathways relating to bioterrorist viral infection and shock.

## 3. Phosphorothiolate-modified oligonucleotides

### 3.1 DNA sequencing-based applications

Searching publications for "phosphorothiolate" and "oligonucleotide" using CAS SciFinder in September 2009 led to only 114 publications in journal and patent databases during 1985–2008, which is far less than the ~5700 items similarly found for "phosphorthioate" and "oligonucleotide." Nevertheless, phosphorothiolate-modified oligonucleotides have proven to be extraordinarily useful in a non-obvious way relative to applications of these compounds pioneered by Engels[25] and Cosstick.[24] This newly established utility is in the field of DNA sequencing, which has rapidly grown in significance both scientifically and commercially. Part of this growth is being driven by comparative genomic sequencing aimed at better understanding genetic diversity. For example, the international 1000 Genomes Project (http://www.1000genomes.org) that began in 2008 will provide a public resource of almost all genetic variants across the human genome with a frequency of 1% or higher, and of genetic variants with even lower frequencies in gene regions. This initial work is scheduled to be completed in 2010.

New sequencing technologies being used for comparative genomics, and other applications, were in part brought about by a NIH funding-initiative beginning in 2004 entitled "Revolutionary Genome Sequencing Technologies—the $1000 Genome." The intent was to fund development of novel technologies that would enable extremely low-cost genomic DNA sequencing. Technologies at that time were said by the NIH to produce desired quality sequence of a human genome (3 Gb = $3 \times 10^9$ bases) for $10 to $50 million. This funding, and significant commercial potential for next-generation sequencing technology, led to a number of new sequencing platforms.[68] Among these, the approach developed by McKernan *et al.*[69] is noteworthy because it very cleverly adapted unique attributes of 3′-S–P–O-5′[24] and 3′-O–P–S-525′[25] linkages in ODNs—namely, automatable synthesis and metal (*e.g.*, silver) ion-mediated P–S bond cleavage—to enable ligase-based sequencing of single-stranded DNA from both directions.[69] This method called "Sequencing by Oligonucleotide Ligation and Detection" (SOLiD) is briefly outlined in Fig. 8 for the forward direction, *i.e.*, proceeding away from the bead to which template DNA is attached. A high-quality human-genome sequence derived from forward-direction SOLiD has been recently reported[70] with an estimated[70] reagent cost of under $30 000—far below the multi-million dollar cost-range for conventional sequencing. Forward-direction 5′-fluorescently labeled 8-mer SOLiD probes depicted in Fig. 8, which are ligated to 5′-phosphorylated primers, employ three universal bases (Z) to reduce probe-degeneracy from $4^8 = 65\,536$ to $4^5 = 1024$. Two-base color-coding of these SOLiD probes is used to increase base-calling accuracy since each base is read twice.[71] In reverse-direction[69] SOLiD, 5′-phosphorylated-3′-labeled probes are ligated to 3′-hydroxyl primers. Metal ion-mediated P–S bond cleavage of 3′-O–P–S-5′ linkages in probes gives a ligation product having a 3′-phosphate that must be removed with phosphatase prior to the next ligation cycle.

A biochemically interesting, alternative strategy for sequencing in the aforementioned reverse direction has been prophetically described by Smith and McKernan[72] wherein polymerases incorporate fluorescently labeled 2′,3′-dideoxy-3′-thio-nucleoside 5′-triphosphates to generate primer-extension products having cleavable phosphorothiolate linkages. A related embodiment supported by data used a fluorescently labeled primer extended by polymerase-mediated incorporation of up to five contiguous 3′-deoxy-3′-(methyldithio)thymidine 5′-triphosphate substrates resulting in 3′-S–P–O-5′ linkages that were cleaved by reaction with 50 μM silver nitrate for 15 min at room temperature. In view of the fact that such disulfides are known to be easily reduced by dithiothreitol, the reported polymerization presumably occurred by means of *in situ* generation of a 3′-thiol for nucleophilic displacement of pyrophosphate. However, others have reported difficulties in synthesizing 2′,3′-dideoxy-3′-thio-nucleoside 5′-triphosphates substrates free of normal dNTPs, which are preferentially incorporated by polymerases and have confounded this sequencing approach.[73,74] Analogous problems obtaining fluorescently labeled dNTPs free of normal dNTPs have been recently reported[75] for single-molecule sequencing.

For the sake of completeness, it should be noted that two alternative sequencing methods[76,77] and arrayed probes[78] using cleavage of phosphorothiolate linkages have been patented.

As a final, historical note it should be mentioned that the possibility of using cleavage of non-bridging PS linkages for sequencing was first reported by Gish and Eckstein in 1987.[79,80] This method was predicated on the observation that PS linkages incorporated into DNA (or RNA) by polymerases

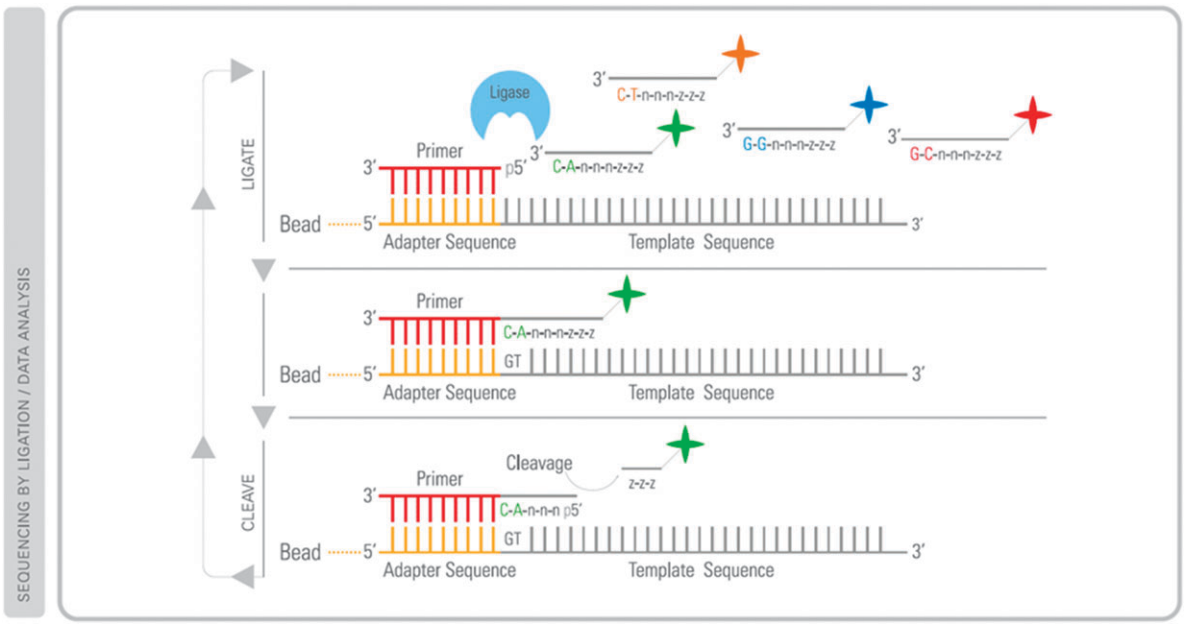



**Fig. 8** Key steps in forward-direction SOLiD: (1) Primers hybridize to the P1 adapter sequence within the library template. (2) A set of four fluorescently labeled di-base probes compete for ligation to the sequencing primer. Specificity of two-base probes is achieved by interrogating every 1st and 2nd base in each ligation reaction. (3) Multiple cycles of ligation, detection and cleavage are performed with the number of cycles determining the eventual read length. (4) Following a series of ligation cycles, the extension product is removed and the template is reset with a primer complementary to the $n-1$ position for a second round of ligation cycles. (5) Five rounds of primer-reset are completed for each sequence tag. Through the primer reset process, each base is interrogated in two independent ligation reactions by two different primers.

are hydrolysed by treatment with 2-iodoethanol in a solution of aqueous ethanol. For DNA sequencing, primed single-stranded M13 DNA was polymerized with the Klenow fragment of DNA polymerase I in the presence of the three normal dNTPs and one α-thio dNTP. This was followed by treatment with 2-iodoethanol, precipitation of the DNA fragments and analysis by polyacrylamide electrophoresis.

### 3.2 RNA-based applications

Cech and Cosstick and coworkers reported[81] a non-phosphoramidite synthetic route to an RNA dinucleotide (IspU) containing a 3′-S–P–O-5′ linkage that was found to be a substrate for T4 polynucleotide kinase, snake venom phosphodiesterase and ribonuclease $T_2$. Base-catalyzed cleavage of the phosphorothiolate bond was accelerated ~2000-fold relative to the case without sulfur, and was attributed to stabilization of the anionic transition state by the polarizable sulfur atom. Shortly thereafter, Piccirilli and coworkers[82] described a 3′-thiophosphoramidite route to RNA with 3′-S–P–O-5′ linkages for characterization of phosphorothiolate bond cleavage reactions that included hydroxide, iodine and silver ion. Use of such compounds for studying pre-mRNA splicing has been reported, together with a review of relevant literature available at that time. Potential applications of 3-phosphorothioate-modified siRNA analogs for RNAi investigations have been recently suggested.[83]

## 4. Perspectives

Based on the foregoing discussion of publications related to automated synthesis of oligonucleotides having PS or phosphorothiolate linkages, several observations can be drawn. Availability of solid-phase DNA synthesis that was adapted to fully automated preparation of fully modified PS-ODNs in 1984 sparked an enormous amount of work aimed at developing nuclease-resistant antisense therapeutics. Approximately 25 years later, this objective is still receiving considerable attention, as evidenced by the relatively large number of ongoing clinical investigations of PS-modified ODNs. Automated methods for synthesis of RNA have been significantly improved following initial commercialization in 1986,[84] with more recent major advances being accelerated by widespread interest in using chemically synthesized siRNA—including PS-modified analogs—for RNAi *in vitro* and as a mechanistic basis for possible therapeutics. In these antisense- and siRNA-driven developments, phosphorothiolate modifications have not been widely investigated due, in part, to unavailability of commercial suppliers of 3′-thio- and 5′-thiophosphoramidite reagents. Hopefully, these reagents will be made available in the near future and thus facilitate investigations of phosphorothiolate-modified DNA and siRNA.

Pursuit of PS- and phosphorothiolate-modified ODNs for basic biochemical studies and possible antisense-based therapy led to unexpected yet extraordinarily useful "spin-off" applications. Immunostimulatory PS-ODNs are now in clinical trials sponsored by several companies, "thioaptamers" are being pursued for various applications, and low-cost, synthetic PS-modified random primer availability has enabled WGA for various types of bioanalytical or genetic analyses.

Finally, and perhaps most exciting, automated synthesis of phosphorothiolate-modified ODNs for ligation and subsequent phosphorothiolate bond cleavage was invented as a

means of ultrahigh-throughput, lower cost DNA sequencing. This novel phosphorothiolate-enabled sequencing methodology, which is now being applied to the 1000 Genomes Project, and is applicable to transcriptome sequencing, will undoubtedly continue to transform the ways by which basic and applied science are carried out to improve life.

Ralph Waldo Emerson's observation, which was quoted at the beginning of this account, that "*the creation of a thousand forests is in one acorn*" does indeed seem to be reflected in the aforementioned "forests" of applications derived from the "acorn" of automating synthesis of phosphorus–sulfur analogs of nucleic acids.



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